CHAPTER 2

Pulmonary administration of small interfering RNA: the route to go?

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ABSTRACT

Ever since the discovery of RNA interference (RNAi), which is a post-transcriptional gene silencing mechanism, researchers have been studying the therapeutic potential of small interfering RNA (siRNA) to treat diseases that are characterized by excessive gene expression. Excessive gene expression can be particularly harmful if it occurs in a vulnerable organ such as the lungs as they are essential for physiological respiration. Consequently, RNAi could offer an approach to treat such lung diseases. Parenteral administration of siRNA has been shown to be difficult due to degradation by nucleases in the systemic circulation and excretion by the kidneys. To avoid these issues and to achieve local delivery and local effects, pulmonary administration has been proposed as an alternative administration route. Regarding this application, various animal studies have been conducted over the past few years. Therefore, this review presents a critical analysis of publications where pulmonary administration of siRNA in animals has been reported. Such an analysis is necessary to determine the feasibility of this administration route and to define directions for future research. First, we provide background information on lungs, pulmonary administration, and delivery vectors. Thereafter, we present and discuss relevant animal studies. Though nearly all publications reported positive outcomes, several reoccurring challenges were identified. They relate to 1) the necessity, efficacy, and safety of delivery vectors, 2) the biodistribution of siRNA in tissues other than the lungs, 3) the poor correlation between *in vitro* and *in vivo* models, and 4) the long-term effects upon (repeated) administration of siRNA. Finally, we present recommendations for future research to define the route to go: towards safer and more effective pulmonary administration of siRNA.

INTRODUCTION

Though substantial obstacles were encountered over the past few decades, gene therapy has maintained the interest of numerous researchers as a potential approach to treat diseases that are caused by genetic dysregulations. Several gene therapy approaches exist (e.g., delivery of DNA into the nucleus, delivery of RNA or antisense oligonucleotides into the cytoplasm, and modification of the genome), and they are distinguished from each other on the basis of their mechanism and outcome (i.e., removing, restoring, or silencing gene expression in cells) [1–3]. For instance, successful DNA delivery produces transgene expression in cells to restore endogenous protein levels, to introduce novel cellular functions, or to produce viral proteins for vaccination purposes [1]. In contrast, cytoplasmic delivery of RNA, such as small interfering RNA (siRNA) and micro RNA (miRNA), can induce post-transcriptional gene silencing through RNA interference (RNAi) (fig. 1) [2]. Lastly, recent advancements in genome-editing technologies have enabled the precise and accurate removal, correction, or insertion of genes by using programmable nucleases (e.g., meganucleases, zinc finger nucleases, transcription activator-like effector nucleases, and CRISPR-associated Cas9 nucleases) [3].

Of special interest is cytoplasmic delivery of miRNA or siRNA because transport into the nucleus is not required to achieve therapeutic effects. Upon entering the cytoplasm, miRNA or siRNA duplexes, which consist of a guide strand and a passenger strand, are loaded into an RNA-induced silencing complex (RISC) precursor [2]. Subsequent guide strand selection occurs, after which the passenger strand is ejected and degraded. Strands that have the lowest 5’ end base-pairing strength are most likely selected as a guide strand. Thereafter, the activated RISC captures messenger RNA (mRNA) molecules that have a complementary base sequence to the guide strand [4]. Then, depending on the associated Argonaute (AGO) protein and the base-pairing match between the activated RISC and target mRNA, mRNA can either be destabilized or cleaved [5]. Destabilization does not necessarily lead to degradation whereas cleaving obviously does. Cleaving only occurs if AGO2 is present in the RISC and if base-pairing is (nearly) perfect. As miRNA generally pairs imperfectly with mRNA, cleaving is usually only observed with siRNA. Another difference between miRNA and siRNA is that miRNA can target multiple genes simultaneously while siRNA only targets a single gene [6]. Although delivery approaches are similar (i.e., miRNA and siRNA are both active in the cytoplasm), siRNA is preferred over miRNA as saturation of miRNA processing enzymes may occur [7]. This might lead to more off-target effects and less efficient gene silencing with miRNA. In addition, the efficacy and safety of

miRNA therapeutics has not yet been explored in clinical trials as only limited research has been carried out so far. Therefore, siRNA currently has a greater therapeutic potential than miRNA as gene silencing is more effective and more specific.

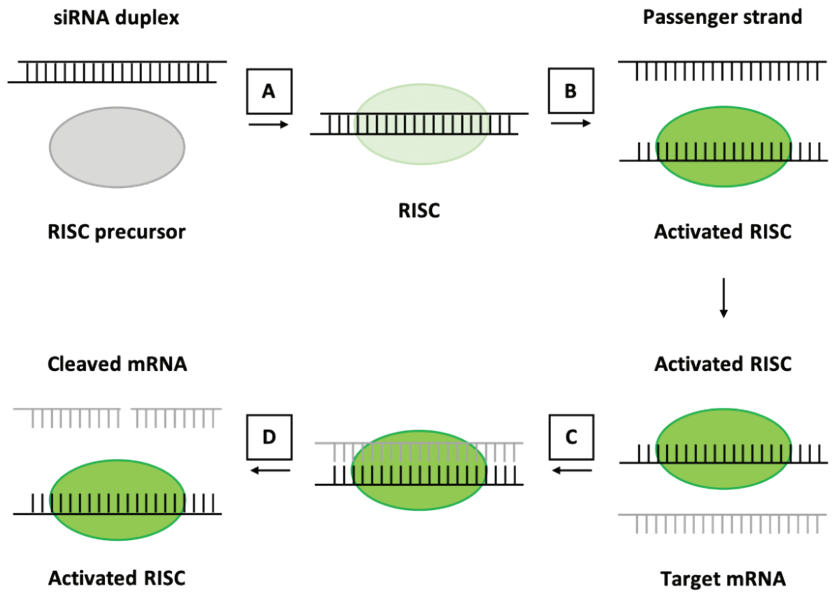

FIGURE 1. siRNA-based RNA interference. Upon internalization of small interfering RNA (siRNA) by cells, association occurs with an RNA-induced silencing complex (RISC) precursor (a). Subsequent guide strand selection occurs and the passenger strand is ejected from the RISC, resulting in activation of the RISC (b). Next, the activated RISC captures messenger RNA (mRNA) that bears a complementary sequence (c). Finally, the mRNA is cleaved and ejected from the activated RISC, which can be reused again (d).

However, unmodified siRNA has a short half-life in the systemic circulation [8]. Firstly, nucleolytic degradation occurs as nucleases are ubiquitous in blood. Secondly, rapid renal clearance occurs because siRNA duplexes (~13 kDa, 20-24 bp) are not retained upon glomerular filtration. As a consequence, parenteral administration is not feasible unless delivery vectors are used to protect the siRNA. Parenteral administration also exposes the whole human body to siRNA which may hamper the delivery efficiency to target tissues or organs. To avoid nucleolytic degradation and renal clearance, local drug administration routes have been proposed which aim for direct delivery to the site where the therapeutic action is desired. Because numerous lung pathologies are caused by excessive transcription of genes (e.g., lung cancer, idiopathic pulmonary fibrosis (IPF), asthma, respiratory

syncytial virus (RSV) infection and influenza infection), pulmonary administration has great potential as it provides the opportunity to directly deliver siRNA to diseased lung tissue in a minimally invasive manner [9,10]. These lung diseases could potentially benefit from the pulmonary administration of siRNA. With respect to pulmonary administration of siRNA in animals, various proof-of-concept studies have been conducted and reported over the past few years.

Therefore, the principal aim of this review is to present a critical analysis of publications that have reported the pulmonary administration of siRNA in animals. This is necessary to determine the feasibility of pulmonary administration for gene silencing purposes and to define the direction for future research. Firstly, we describe background information on fundamental topics (e.g., lungs, pulmonary administration, and delivery vectors) to provide insight into relevant concepts and technologies. Secondly, we present and discuss publications that have reported the administration of siRNA via the pulmonary route in animals. Recent successes as well as current challenges are identified. Lastly, we provide recommendations for future research based on current challenges as this could, ultimately, prompt further research.

RESPIRATORY SYSTEM

Anatomy and physiology

The lungs are fascinating organs and they belong to the respiratory system (fig. 2). The entire respiratory system includes the upper airways (i.e., the nasal cavity, oral cavity, pharynx, and larynx) and the tracheobronchial tree (i.e., the trachea, bronchi, bronchioles, alveolar ducts, alveolar sacs, and alveoli) [11]. Importantly, the tracheobronchial tree is commonly known as “the lungs”. Humans have two lungs which are both located in the thoracic cavity; the right lung consists of three lobes and the left lung consists of two lobes. Extensive branching is observed in the tracheobronchial tree and it is characterized by asymmetrical dichotomy that occurs for approximately 23 generations. This branching substantially increases the internal surface area of the lungs (~100 m^2) to enable effective gas exchange [12]. From a functional perspective, the respiratory system can also be divided in two parts: the conductive zone (i.e., the upper airways, trachea, bronchi, and terminal bronchioles) and the respiratory zone (i.e., the respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli). The conductive zone conditions and conducts atmospheric air, whereas the respiratory zone facilitates the exchange of oxygen and carbon dioxide. These processes are assisted by an extensive network of arteries, veins, and capillaries.

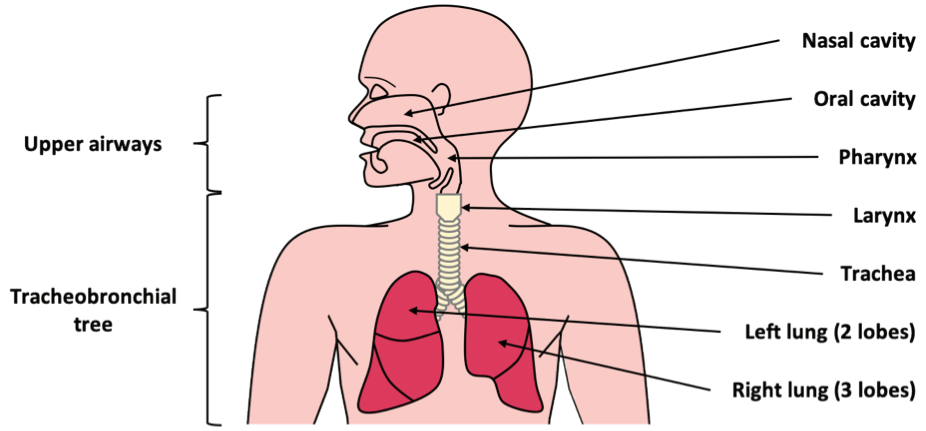

FIGURE 2. Respiratory system. The respiratory system consists of the upper airways (i.e., nasal cavity, oral cavity, pharynx, and larynx) and the tracheobronchial tree (i.e., trachea, the bronchi, bronchioles, alveolar ducts, alveolar sacs, and alveoli).

With respect to blood supply, lungs receive blood from the bronchial circulation and the pulmonary circulation [13]. The high-pressure bronchial circulation supplies nutrients to the lungs as it receives oxygen-rich blood from the bronchial arteries, which arise from the aorta. In contrast, the low-pressure pulmonary circulation receives oxygen-depleted blood from the pulmonary arteries, which emerge from the right ventricle of the heart. Both circulations complement each other. However, the bronchial circulation and the pulmonary circulation are connected by arteriovenous anastomoses which drain predominantly into the pulmonary vein and partially into the bronchial vein [14]. Furthermore, the lungs form a remarkably thin interface of approximately 0.2-0.7 μm between the alveolar lumen and capillary lumen [15]. This interface, together with the large surface area of the lungs, enables rapid and effective physiological respiration. Physiological respiration is an essential homeostatic process and refers to the uptake of oxygen into the blood and the removal of carbon dioxide from the blood [13].

Because of their importance for homeostasis and vulnerability to diseases, the lungs apply several strategies to protect the body from physical, biological, and chemical threats. For example, physical threats (e.g., the deposition of atmospheric particles) are counteracted by mucociliary clearance which occurs in the conducting zone of the tracheobronchial tree [15]. In this zone, inhaled particles can be deposited on mucus that covers the airways. Ciliated epithelial cells subsequently transport mucus in an upward direction towards the pharynx where mucus is either swallowed or expectorated. Furthermore, biological threats (e.g.,

the invasion of infectious agents) are minimized through constant surveillance of lung tissue by antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages [16]. Additional protection against pathogens is provided by the pulmonary mucus which contains various antimicrobial, antioxidant, and anti-inflammatory substances [17]. Lastly, chemical threats (e.g., the introduction of toxic substances) are reduced by biotransformation enzymes which are present in the lungs, albeit in a lower amount than in the intestines or liver. These enzymes perform phase 1 reactions (e.g., oxidation, reduction, and hydrolysis) and phase 2 reactions (e.g., glucuronidation, sulfation, methylation, acetylation, and glutathione conjugation) [18]. However, despite these protection strategies, the lungs remain vulnerable.

Pathologies and targets

As mentioned earlier, the lungs are prone to a number of specific diseases that contribute substantially to the global burden of disease in terms of hospitalization, financial costs, and deaths [9]. If lung diseases are characterized by a genetic dysregulation which necessitates gene silencing, pulmonary administration of siRNA could be used to inhibit the translation of endogenous or exogenous mRNA. Obviously, this requires extensive knowledge on the pathogenesis of diseases to accurately identify mRNA targets. Preferably, an mRNA target should be specifically related to a disease to minimize off-target effects and it should be substantially involved in the pathogenesis to maximize therapeutic effects. Therefore, this section aims to describe mRNA target selection of the following three common lung diseases: lung cancer, IPF, and influenza.

Currently, lung cancer remains one of the most frequent cause of cancer-related deaths worldwide and it is classified as either small-cell lung cancer or non-small-cell lung cancer [19]. In 2016, 224,390 new lung cancer cases and 158,080 lung cancer deaths were predicted in the United States alone [20]. Therefore, there is a great medical need for effective treatments because this could significantly decrease the burden of disease. As cancer is characterized by excessive transcription, siRNA against serine-threonine protein kinase (*AKT1*) mRNA could be a potential target to treat non-small cell lung cancer because it is involved in angiogenesis and cell metabolism, proliferation, survival, and growth [21]. Several *in vitro* and *in vivo* studies have demonstrated the feasibility of this strategy [21–26].

IPF is another common lung disease and it is characterized by abnormal extracellular matrix (ECM) remodeling after acute lung injury [27]. In healthy lungs, there is a dynamic balance between the production, deposition, and degradation of ECM. However, this balance is lost in IPF, and ECM production is favored over ECM degradation. The degradation of ECM is controlled by matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) [28]. MMPs degrade ECM, whereas TIMPs inhibit MMPs. In IPF patients, TIMP expression is increased which results in an increased inhibition of MMP activity and, consequently, the preservation of a non-degrading microenvironment [29]. Multiple TIMPs (i.e., TIMP-1, TIMP-2, TIMP-3, and TIMP-4) have been identified, each with its specific localization in lung tissue [28]. Therefore, a possible approach to treat IPF with RNAi would be to administer siRNA against *TIMP* mRNA via the pulmonary route as this could potentially shift the balance of ECM remodeling towards degradation.

Lastly, influenza is an infectious disease caused by the influenza virus which enters the body via the respiratory system [30]. Seasonal influenza epidemics already result in countless hospitalizations, and influenza pandemics pose even more danger to humans and society as a whole. Especially young, old, and immunocompromised persons are at risk as shown by increased morbidities and death rates [31]. Though much research has focused on the development of vaccines, they only have a limited efficacy and specificity due to high mutation rates of the influenza virus. Novel treatments are therefore urgently needed. Using siRNA against mRNA that originates from highly conserved regions of the viral genome could offer a potential approach to treat patients who suffer from influenza infections and to decrease the transmission rate of the virus between humans. For instance, inhibiting the translation of nucleocapsid-encoding mRNA highly restricts the viral replication as viral proteins cannot assemble into complete virus particles [32]. Moreover, studies have shown that intranasal administration of siRNA against nucleoprotein (NP) of influenza can significantly inhibit viral replication and improve survival rates in mice upon infection with a lethal dose of influenza [32,33].

PULMONARY ADMINISTRATION

Although pulmonary administration of siRNA could potentially be used to treat lung diseases, two substantial obstacles should be overcome. First of all, siRNA should be transported to a target site in the lungs where therapeutic effects are desired. This requires the selection of appropriate inhalation devices such

as nebulizers, metered-dose inhalers (MDIs), or dry powder inhalers (DPIs). Thereafter, siRNA needs to be transported into target cells in order to exert biological effects in the cytoplasm. Internalization, however, consists of initial endocytosis and subsequent endosomal escape which remains difficult to achieve and may necessitate the use of delivery vectors. Therefore, this section aims to provide more insight into relevant transport mechanisms and potential inhalation devices.

Transport mechanisms

Successful pulmonary administration of siRNA requires initial particle deposition near targeted lung cells and subsequent internalization. Deposition of particles in the upper airways and the tracheobronchial tree is controlled by several mechanisms, including inertial impaction (diameter-dependent), sedimentation (diameter-dependent), diffusion (diameter-dependent), interception (shape-dependent), and electrostatic precipitation (charge-dependent) [34,35]. An important variable for particle deposition is the aerodynamic diameter, which is calculated from the geometric diameter and the particle density and shape [36]. Particles with an aerodynamic diameter greater than 5 µm are more frequently deposited in the throat because of inertial impaction, whereas particles in the range of 0.1-1 µm are simply exhaled due to limited deposition. In contrast, particles with an aerodynamic diameter of 1-5 µm are also deposited in the alveolar region [37,38]. Though particle deposition is dependent on the aerodynamic diameter, particles are never exclusively deposited in specific regions. For example, deposition of particles always occurs to a certain extent in the upper airways even when alveolar regions are targeted. Nevertheless, deposition of siRNA at target cells alone is not sufficient to produce therapeutic effects.

After deposition, internalization (i.e., endocytosis and endosomal escape) of siRNA is necessary before gene silencing can occur [39]. Endocytosis describes the uptake of extracellular substances by cells and can be clathrin-dependent, caveolin-dependent, or clathrin/caveolin-independent. In addition, endocytosis also includes macropinocytosis. The internalization efficiency and selectivity may be improved by conjugating siRNA with a ligand that binds to particular receptors (e.g., integrins, g-protein coupled receptors, and receptor tyrosine kinases) which are expressed on the surface of targeted cells [39]. Upon successful endocytosis, siRNA-containing endosomes are formed which mature from early to late endosomes and finally to lysosomes [40]. The endosomal pH decreases substantially during the maturation process to promote degradation of its

contents. As a consequence, endosomal escape of siRNA is necessary to achieve cytoplasmic delivery. Recently, it was shown that endosomal escape of siRNA into the cytoplasm is extremely limited (1-2%) [41]. Various approaches have been proposed to improve endosomal escape, such as pore formation, pH-buffering effects, and fusion in the endosomal membrane [40]. Nonetheless, transport of siRNA into the cytoplasm remains complicated, despite these more advanced approaches.

Inhalation devices

As mentioned previously, pulmonary administration of siRNA requires the selection of an appropriate inhalation device to first facilitate the deposition of siRNA in lung tissue. Various types of inhalation devices exist and they exploit different mechanisms to generate inhalable aerosols. Inhalation devices are classified as nebulizers, soft mist inhalers, MDIs, and DPIs [42]. Nebulizers generate liquid aerosols by using either compressed air or ultrasonic power to disperse drug solutions (or suspensions) into small droplets with a particular size range [43]. Soft mist inhalers differ fundamentally from classical nebulizers as they produce a mist of fine aqueous droplets by a vibrating mesh, Rayleigh breakup, or impinging jets [44]. MDIs contain drugs formulated with a liquefied propellant to keep the mixture under pressure. Upon use, a predetermined amount of drug formulation is forced through a nozzle to produce high-velocity liquid aerosols. In contrast, DPIs generate inhalable dry powder aerosols by directing airflow, which is generated by the user upon inhalation, through a drug powder. This causes dispersion of the drug powder according to deagglomeration principles and allows for subsequent inhalation of the aerosolized drug [36]. Each type of inhalation device has its strengths and weaknesses; and certainly not all inhalation devices are suitable for pulmonary administration of siRNA [43,44]. Furthermore, selecting an inhalation device should also depend on the nature of the disease which is ought to be treated (e.g., acute or chronic).

Ultrasonic and jet nebulizers, for example, are unlikely to be optimal inhalation devices for siRNA due to an unfavorable delivery efficiency, drug stability, usability, portability, and cost-profile [45,46]. The delivery efficiency, which refers to the amount of drug which is actually deposited in the lung, is limited as not all drug solution (or suspension) is aerosolized during nebulization. Furthermore, siRNA is exposed to high shear forces upon nebulization. Consequently, the potency of siRNA may be reduced, though delivery vectors could protect siRNA from nebulization-induced degradation. In contrast to MDIs and DPIs, nebulizers require long

administration times, and they demand frequent disinfection to prevent microbial contamination. Disposable nebulizers may be used to avoid regular cleaning but this could increase the costs. The high intrinsic costs of the nebulizers may increase further as more siRNA is required to account for low delivery efficiencies and nebulization-induced degradation. Nebulizers also have a limited portability due to their size and weight. Soft mist inhalers address several of these issues [47]. However, they use liquid formulations which are undesirable due to risks of contamination with nucleases or microorganisms. To that end, MDIs may not be ideal either. Furthermore, MDIs require a correct inhalation technique and coordination which is difficult to achieve by children. The high-velocity aerosols generated by MDIs are also likely to cause particle deposition in the throat area, thereby limiting the delivery efficiency. DPIs do not have these disadvantages and are therefore highly preferred. DPIs do require the development of a dry powder formulation. More information on the development of inhalable siRNA powders can be found in an excellent review written by Chow and Lam (2015) [48].

DELIVERY VECTORS

To protect siRNA from degradation and to promote cellular internalization of siRNA, delivery vectors can be used. Extensive research efforts have resulted in numerous types of vectors (fig. 3). Vectors can be classified as viral (e.g., lentivirus, adenovirus, and adeno-associated virus vectors) or non-viral (e.g., polymer-based and lipid-based systems). Each type of vector has its advantages and disadvantages with respect to the efficacy and safety, which in this case should be considered within the context of pulmonary administration. Therefore, this section describes commonly used vector types and discusses their advantages and disadvantages.

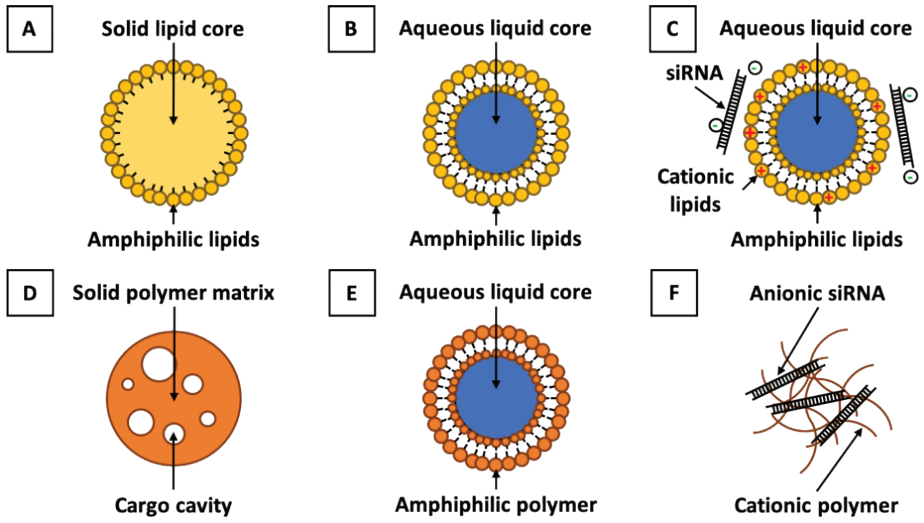

FIGURE 3. Non-viral delivery vectors. Lipid-based systems include (a) solid lipid nanoparticles, (b) liposomes, and (c) lipoplexes. Polymer-based systems include (d) solid polymer nanoparticles, (e) polymersomes, and (f) polyplexes.

Viral

Over the course of time, viruses have evolved to enter cells as efficiently as possible. As a result, the viral machinery can be exploited to improve the internalization of genetic material. Several viral vectors are available for these purposes such as the lentivirus (LV), adenovirus (AV), and adeno-associated virus (AAV) [49–51]. Though these vectors can enter dividing and non-dividing cells, they have major differences between each other. For instance, the enveloped LV (*retroviridae* family) carries a positive single strand RNA genome. Furthermore, the LV has a limited cargo capacity of ~8 kb, despite having a size of ~80-100 nm. In contrast, the non-enveloped AV (*adenoviridae* family) contains a linear double strand DNA genome and it has a large cargo capacity of ~37 kb, though it has a size of ~100 nm. Finally, the non-enveloped AAV (*parvoviridae* family) has a single strand DNA genome, a relatively small size of 25 nm, and a limited cargo capacity of only ~4.7 kb.

In addition, viral vectors often contain (plasmid) DNA as they have the ability to access the nucleus of cells. This (plasmid) DNA could encode short hairpin RNA (shRNA) of ~70 bp and produce long-term gene silencing [52,53]. shRNA is an RNA molecule with self-complementary sites which anneal to form a tight hairpin turn. However, shRNA requires additional preliminary processing to turn into siRNA.

Firstly, upon successful delivery into the nucleus, (plasmid) DNA is transcribed to shRNA by RNA polymerase II or III. Transcribed shRNA is then exported from the nucleus via exportin 5, which is a karyopherin that mediates transport of molecules between the nucleus and cytoplasm. Next, shRNA is loaded into a RISC precursor which contains accessory proteins, such as Dicer and AGO. After processing shRNA to duplex siRNA by Dicer, the passenger strand is ejected and the RISC is activated to induce long-term post-transcriptional gene silencing through RNAi mechanisms, as described earlier.

Although viral vectors have various advantages, they also have disadvantages regarding safety, manufacturability, and efficacy. Firstly, viral vectors such as the LV and AAV may integrate their viral genomes into the host genome. Depending on the integration site, this could have serious consequences, such as tumorigenesis [51]. Secondly, it can be challenging to produce viral vectors in a consistent manner and in a large quantity. As a result, the clinical applicability as well as the potential approval by regulatory authorities may be hampered [49]. Lastly, viral vectors can be highly immunogenic. This can be especially problematic when targeting mucosal areas and delicate tissues like the lungs which contain many immune cells. For example, persons may have pre-existing antibodies against vectors such as the AV. Upon pulmonary administration, such vectors can be rapidly neutralized by the immune system. These disadvantages, however, may be tackled in the (near) future to enable long-term gene silencing in humans (e.g., by selecting rare or synthetic viral vector serotypes to minimize the amount of people who have pre-existing antibodies against a particular vector).

Non-viral

As an alternative, non-viral vectors can be used to address the disadvantages associated with viral vectors. Of special interest are lipid-based and polymer-based systems as they are most commonly used [54,55]. Several strategies can be used to produce these vectors (e.g., by encapsulation or complexation). For instance, siRNA can be encapsulated into a lipid-based or polymer-based solid matrix, yielding solid lipid nanoparticles (SLNs) or solid polymer nanoparticles (SPNs). Furthermore, encapsulation of aqueous siRNA into vesicles is possible by using amphiphilic lipids or amphiphilic polymers to produce liposomes or polymersomes, respectively. Lastly, complexation of negatively charged siRNA with positively charged lipids or polymers yields lipoplexes or polyplexes, respectively. However, the production of non-viral vectors is not limited to these strategies and materials. Though efficacy and safety concerns still apply, other approaches and modifications are available as

well, such as the use of dendrimers, inorganic nanoparticles, and cell-penetrating peptides [56].

Physicochemical properties of non-viral vectors can be modified to improve the biocompatibility, to increase internalization, or to achieve active targeting. For example, research has shown poly(lactic-co-glycolic acid) (PLGA) nanoparticles can be coated with chitosan to successfully change the surface charge from negative to positive, without dramatically changing the particle size distribution [57]. This could result in an enhanced internalization efficiency due to electrostatic attraction as cell membranes are negatively charged. Vectors should remain biocompatible or toxic side effects may manifest. The biocompatibility could be improved by adding polyethylene glycol (PEG) groups to vector surfaces. Consequently, vectors have been shown to exhibit less non-specific interactions with cells, though this modification has also been shown to impair endosomal escape [58]. Recently, active targeting has received more attention as well, and it involves the attachment of affinity ligands (e.g., antibodies, peptides, or small molecules) to the vector surface. Affinity ligands bind to receptors that are expressed (in excess) on the surface of target cells. Coating vectors with affinity ligands could therefore improve the uptake of vectors by particular cells [54].

Despite nearly limitless modifications, researchers have identified serious challenges concerning the efficacy and safety of non-viral vectors. Safety issues arise from a limited biocompatibility and biodegradability. Biocompatibility issues develop when non-viral vectors induce adverse events in biological tissues. For instance, non-viral vectors are often composed of cationic polymers (e.g., polyethylenimine (PEI) or its derivatives) or lipids (e.g., DOTMA, DOTAP, and DOSPA). These components interact with negatively charged components in serum which can lead to adverse events [9,54]. Furthermore, biodegradability issues occur if vectors persist in biological tissues. Importantly, repeated administration of non-biodegradable or slowly-degrading vectors could lead to chronic build-up of material (especially in the alveolar region) [55]. This could be particularly dangerous if vectors are administered via the pulmonary route. In addition, non-viral vectors are less efficiently internalized than viral vectors, as viral vectors have specialized machinery for such purposes. Thus, developing non-viral vectors also proves to be difficult.

ANIMAL STUDIES

Though extensive research efforts have focused on the development of delivery vectors for siRNA, it remains important to validate their necessity, efficacy, and safety in animal studies. Over the past few years, fifteen publications appeared which reported the pulmonary administration of siRNA in animals (table 1). Only mice have been used as an animal model. These animal studies will be reviewed here, according to the type of delivery vector that was used, to identify current challenges. In addition, there will be a brief discussion on the predictive value of animal models within the context of pulmonary administration.

TABLE 1. Pulmonary administration of siRNA in mice.

Vector	Vector system	siRNA target	Outcome[a]	Reference
None	N/A	Xcl1	+	Rosas-Taraco et al. (2009) [59]
	N/A	Luciferase	-	Moschos et al. (2011) [60]
Viral	Lentivirus	Aimp2-dx2	+	Hwang et al. (2010) [61]
	Lentivirus	Pdcd4	+	Hwang et al. (2013) [24]
Non-viral	Polymer-based	Akt1	+	Jere et al. (2008) [26]
	Polymer-based	Akt1	+	Xu et al. (2008) [25]
	Lipid-based	N/A[b]	N/A[c]	Garbuzenko et al. (2009) [62]
	Polymer-based	Akt1	+	Jiang et al. (2009) [63]
	Polymer-based	Wt1	+	Zamora-Avila et al. (2009) [64]
	Polymer-based	Egfp	+	Nielsen et al. (2010) [65]
	Polymer-based	Egfp	+	Beyerle et al. (2011) [66]
	Inorganic	N/A[b]	N/A[c]	Taratula et al. (2011) [67]
	Polymer-based	Luciferase	+	Okuda et al. (2013) [68]
	Lipid-based	Mcl1	+	Shim et al. (2013) [69]
	Polymer-based	Ptprc	+	de Backer et al. (2015) [70]

[a] Gene silencing effects were observed (+) or not observed (-).
[b] Fluorescent non-targeting siRNA was used here.
[c] Gene silencing effects were not observed as these publications only reported biodistribution results.

No vectors

Two publications described the pulmonary administration of siRNA in mice without using a delivery vector (naked siRNA). Rosas-Taraco et al. (2009) administered siRNA against *Xcl1* (mRNA encoding the chemokine XCL1) in mice, which were infected for 60 days with *mycobacterium tuberculosis*, by intratracheal instillation [59]. Transient but significant post-transcriptional gene silencing was observed during the first five days which suggests successful internalization of siRNA by $CD8^+$ and $CD4^+$ T lymphocytes as they are the main XCL1 producers. In addition, the authors showed fluorescently labeled siRNA remained confined to the lungs. However, long-term effects (i.e., 180 days after administration) included overcompensation of XCL1 production. In contrast, Moschos et al. (2011) observed no post-transcriptional gene silencing in luciferase-expressing mice upon intratracheal administration of siRNA against mRNA which encoded luciferase [60]. Furthermore, fluorescently labeled siRNA was shown to partially accumulate in the bladder. These findings suggest naked siRNA was transported into the systemic circulation and subsequently excreted via the kidneys. This is not surprising considering the small size of siRNA that allows for absorption into the systemic circulation upon inhalation. Collectively, these studies provide inconclusive evidence of successful pulmonary administration of naked siRNA.

Viral vectors

Though viruses have great potential, only two publications reported the use of viral vectors. Hwang et al. (2010) has shown *Pdcd4* mRNA levels were significantly decreased in mice after inhalation of a liquid aerosol containing LV vectors encoding shRNA [61]. Furthermore, it was shown that inhibition of PDCD4 synthesis increased tumorigenesis by increasing the levels of anti-apoptotic proteins. This study aimed to achieve gene silencing via inhalation to identify the function of PDCD4. However, no results were reported with respect to the immunogenicity and cytotoxicity of LV vectors. More recently, Hwang et al. (2013) described inhalation of a liquid aerosol containing LV vectors encoding shRNA against *Aimp2-dx2* mRNA successfully decreased tumorigenesis in $AIMP2^{+/-}$ mice [24]. Although the actual gene silencing efficacy in cells was not determined, hyperplasias were less frequently detected in mice treated with targeting shRNA (against *Aimp2-dx2*) as opposed to mice treated with non-targeting shRNA (negative control). Interestingly, both studies indicated successful gene silencing after inhalation of LV-based vectors, which contained (plasmid) DNA encoding shRNA. However, other viral vectors (e.g., AV and AAV) have not been studied yet. Furthermore, it remains unclear if viral vectors are safe to use and if they are immunogenic.

Non-viral vectors

Non-viral vectors are the most popular as eleven publications (out of fifteen) report their use. Jere et al. (2008) was the first to report preliminary results suggesting that pulmonary administration of biodegradable poly(β-amino ester) (PAE) nanoparticles containing siRNA against *Akt1* decreased AKT1 protein levels *in vivo* [26]. No comprehensive analysis regarding the safety was provided, although the delivery vector was shown to efficiently deliver siRNA into cells with a limited toxicity *in vitro*. Similarly, Xu et al. (2008) also described the use of PAE nanoparticles which contained siRNA [25]. This publication represented follow-up research based on findings of Jere et al. (2008) and confirmed that *Akt1*-targeting siRNA could significantly inhibit lung tumor progression in mice upon pulmonary administration. However, no data was shown with respect to the *in vivo* cytotoxicity of the delivery vector. Although gene silencing was not studied upon pulmonary administration, Garbuzenko et al. (2009) reported results on the biodistribution of fluorescently labeled siRNA encapsulated in DOTAP liposomes [62]. According to their results, the majority of siRNA remained in the lungs, though small amounts were eventually detectable in the liver and the kidneys.

Jiang et al. (2009) also reported lung tumor progression was significantly decreased in mice upon inhalation of liquid aerosols containing siRNA against *Akt1* mRNA, as shown by a lower incidence of tumors and a smaller size of tumors in comparison to the control groups [63]. In this case, folate-chitosan-grafted PEI nanoparticles were used as a delivery vector. Importantly, folate-chitosan-grafted PEI nanoparticles were less cytotoxic and had a greater transfection efficiency than PEI (25k) nanoparticles, as determined *in vitro*. A study by Zamora-Avila et al. (2009) described a different approach as the authors complexed plasmid DNA, encoding shRNA that targeted Wilms' tumor gene 1 (*Wt1*) mRNA, with PEI (25k) [64]. After inhalation, the DNA/PEI polyplexes were shown to significantly decrease the size and amount of lung tumor foci in mice. Though the authors did not study the cytotoxicity of the complexes, previous research demonstrated PEI (25k) is cytotoxic [9]. Furthermore, Nielsen et al. (2010) reported that *Egfp*-targeting siRNA in chitosan nanoparticles was successfully delivered in mice by intratracheal instillation to reduce EGFP expression [65]. Naked siRNA was also administered via the same procedure but produced no observable effects. This finding agrees with Moschos et al. (2011). In addition, the authors showed chitosan nanoparticles with fluorescently labeled siRNA were deposited in the conducting and respiratory zone of the airways.

More recently, Beyerle et al. (2011) demonstrated intratracheal administration of polyplexes, which were composed of *Egfp*-targeting siRNA and PEI-PEG polymers, induced gene silencing in mice which expressed EGFP [66]. The authors also showed low molecular weight PEI (8.3k) was less cytotoxic than high molecular weight PEI (25k). PEGylation of the polyplexes was shown to reduce the cytotoxicity as well. However, the addition of PEG groups substantially increased the immunogenicity of the polyplexes. Taratula et al. (2011) reported fluorescently labeled mesoporous silica nanoparticles (MSNs) were mainly present in the lungs (~73%) of mice upon inhalation of liquid aerosols, though eventually they were also detected in the liver (~17%) and kidneys (~9%) [67]. However, the authors did not investigate the actual mRNA knockdown *in vivo*. Next, Okuda et al. (2013) described that siRNA against mRNA encoding luciferase was successfully incorporated into chitosan nanoparticles which produced gene silencing effects upon intratracheal administration in mice [68]. Interestingly, the authors reported gene silencing effects of siRNA were higher with a dry powder formulation than with a liquid formulation. Higher local concentrations of siRNA could have been achieved because the dry powder formulation dissolved in pulmonary lining fluid without any further dilution, as opposed to liquid formulations which contained a solvent. The biodistribution of siRNA-chitosan nanoparticles was also studied. According to the results, fluorescently labeled siRNA was not only present in the lungs but also in other organs, such as the liver and the kidneys.

Shim et al. (2013) published that cationic lipoplexes could significantly increase the *in vivo* internalization of siRNA, which targeted myeloid cell leukemia sequence 1 (*Mcl1*) mRNA [69]. The authors tested several formulations and EDOPC/cholesterol-based lipoplexes were shown to exhibit the highest transfection efficiency and the lowest cytotoxicity. Intratracheal administration of this formulation was shown to reduce tumorigenesis in the lungs of mice. However, no correlation with respect to the transfection efficiency and the cytotoxicity was observed between results of *in vitro* and *in vivo* experiments. Lastly, de Backer et al. (2015) recently demonstrated intratracheal administration of *Ptprc*-targeting siRNA incorporated in pulmonary surfactant-coated dextran nanogels generated significant gene silencing effects in resident alveolar macrophages of mice, whereas naked siRNA exhibited no effects [70]. Collectively, these studies illustrate benefits of using non-viral delivery vectors to enhance the delivery of siRNA, though they also demonstrated cytotoxicity and immunogenicity issues.

Animal models

As mentioned previously, only mice were used in the aforementioned studies. Mice are often used in early animal studies for logistical and economic reasons as they are small, reproduce quickly, and cost little. However, the predictive value of mice experiments should not be overestimated. Anatomical, histological, and physiological differences between species are not only present in the upper airways but also in the tracheobronchial tree (table 2) [71–73]. This hampers the extrapolation of results to humans. For instance, pharmacokinetic properties such as particle deposition and mucociliary clearance are different between species and this affects the dose that remains active in the lungs. Furthermore, it is of great importance to consider the type of disease; animal models should mimic the clinical indication (e.g., asthma, lung cancer, or pulmonary fibrosis) as much as possible. To that end, higher order animals such as dogs and monkeys are expected to provide more relevant *in vivo* data than mice regarding the pulmonary administration of siRNA [74]. Nonetheless, aside from ethical considerations, the use of higher order animals would come at a substantially higher cost. Therefore, researchers should always make a rational selection regarding the animal species they use in their experiments.

TABLE 2. Species differences of commonly used laboratory animals. Adapted from [72,73,75].

Species (weight)	Lung weight (g)	Alveolar area (m^2)	Branching (type)	Respiratory rate (min^{-1})	Ventilation (L/min)
Mouse (0.02 kg)	0.12	0.07	Monopodial	163	0.025
Rat (0.25 kg)	1.5	0.4	Monopodial	85	0.12
Rabbit (2.5 kg)	18	5.8	Monopodial	51	0.8
Monkey (5 kg)	33	-	Monopodial	38	1.67
Dog (10 kg)	100	40.7	Monopodial	23	1.50
Humans (70 kg)	1000	143	Dichotomous	12	7.98

TOWARDS PROGRESS

Challenges

So far, only limited research has been published regarding the pulmonary administration of siRNA in animals. Though the proof of concept was clearly demonstrated as nearly all studies reported positive outcomes, several reoccurring issues could be identified. For instance, it is currently not known if delivery vectors are necessary to achieve gene silencing *in vivo* as positive and negative effects were reported upon pulmonary administration of naked siRNA. Not having to use delivery vectors would be beneficial as it partially removes toxicity issues. Next, it remains unclear what physicochemical properties of delivery vectors favor successful delivery of siRNA into cells. Unfavorable properties are also largely unknown due to a tendency of journals to publish only positive results instead of negative results. Moreover, most delivery vectors are cytotoxic which hampers their clinical use, as shown by *in vitro* viability data. Biocompatibility and biodegradability of delivery vectors are particularly important as the lungs are vulnerable to damage. One study reported a decreased cytotoxicity upon addition of PEG groups but at the cost of a substantially increased immunogenicity [66]. Another challenge is to constrain siRNA to the lungs because various publications reported extensive absorption into the systemic circulation and subsequent deposition in the liver and kidneys. Furthermore, authors observed a poor correlation between *in vitro* and *in vivo* data regarding the internalization and cytotoxicity of delivery vectors which complicates the selection of delivery vectors for *in vivo* experiments. Lastly, long-term effects upon (repeated) siRNA administration are not well understood. Interestingly, Rosas-Taraco et al. (2009) reported preliminary evidence that overcompensation of target gene expression occurred as a result of siRNA administration. These challenges should be addressed to further improve the therapeutic potential of pulmonary siRNA administration.

Perspectives

Pulmonary administration of siRNA, to treat diseases that are caused by genetic dysregulations, has been successfully demonstrated in mice. However, some challenges were identified, as discussed previously. To address these challenges, recommendations for future studies will be provided. First of all, it is currently unknown if delivery vectors are truly necessary to achieve sufficient gene silencing *in vivo*. Animal studies that focus on delivery vectors for pulmonary administration of siRNA should include naked siRNA to verify if such vectors are necessary. Next, (un)favorable physicochemical properties of delivery vectors should be studied to identify how they affect the efficacy and safety. Special attention should also be

given to the biodegradability and biocompatibility of delivery vectors. A design of experiment (DOE) approach could be used for this purpose. Additionally, studies should include data regarding the immunogenicity of siRNA delivery vectors. Pro-inflammatory effects, for example, can be studied by detecting cytokine release in broncho-alveolar lavage fluid (BALF) [66]. Furthermore, it is currently not fully understood what factors could limit the absorption of vectors into the systemic circulation. This requires more biodistribution studies to identify physicochemical properties that affect the systemic availability of siRNA. The low predictive value of *in vitro* models should also be addressed. Novel methods are desired to accurately predict the internalization of siRNA and the cytotoxicity of vectors. *Ex vivo* technologies (e.g., use of precision-cut lung slices) could be useful as they resemble target tissues more closely than cell cultures [76]. Lastly, long-term effects of repeated siRNA administration via the pulmonary route should be studied to determine if adverse effects occur, such as the overcompensation of target gene expression.

Aside from the previously mentioned suggestions, there is also another aspect that research should focus on. Limited work has been carried out with respect to the development of inhalable siRNA formulations. Nearly all animal studies reviewed in this article used liquid formulations. Although liquid formulations are relatively easy to prepare, they are prone to contamination with nucleases, which quickly degrade siRNA. This makes liquid siRNA formulations undesirable, especially for long-term storage [48]. As a consequence, dry powder formulations of siRNA are greatly desired due to the decreased risk of siRNA degradation by nucleases. Moreover, DPIs (i.e., the type of delivery device that is used for inhalation of dry powder formulations) have multiple advantages over MDIs and nebulizers with respect to the delivery efficiency, usability, portability, and cost-profile. Several techniques can be used to produce an inhalable dry powder formulation for siRNA, such as spray drying and spray freeze drying [48]. Although both techniques produce micron-sized particles, spray drying generates spherical or raisin like particles and spray freeze drying creates large porous particles. As only a few articles have appeared on this subject, more research should focus on the development of siRNA microparticles that are suitable for inhalation [77–79]. Furthermore, it would be especially interesting to study the effects of inhalable siRNA powder without delivery vectors.

CONCLUSIONS

Since its discovery, post-transcriptional gene silencing by RNAi has been of great interest as a potential treatment for diseases that are characterized by the overexpression of particular genes. RNAi limits the overexpression of genes by inducing either degradation of mRNA or destabilization of mRNA. This can greatly decrease the extent of mRNA translation by ribosomes. As overexpression of genes can be especially harmful in vulnerable organs, RNAi could provide an approach to treat various lung diseases such as lung cancer, pulmonary infections, and inflammatory diseases. However, targeting siRNA to lung tissue is difficult. Upon parenteral administration siRNA is degraded by nucleases in the serum and excreted by the kidneys. Consequently, pulmonary administration has been suggested as an alternative administration technique because local delivery can be achieved. Therefore, by reviewing publications that reported pulmonary administration of siRNA in animals, this review aimed to determine the feasibility of this administration route and to subsequently define the directions for future research.

Fifteen publications have appeared about administration of siRNA via the pulmonary route, and nearly all of them reported positive outcomes; pulmonary administration is a promising administration route for the delivery of siRNA. Despite positive results, however, several challenges could be identified. For instance, it is not known if delivery vectors are absolutely necessary to achieve RNAi *in vivo* as animal data is inconclusive. Also, little is known regarding long-term effects that may occur after repeated pulmonary administration of siRNA. Furthermore, not enough is known with respect to (un)favorable physicochemical properties of vectors and how these properties affect the biodistribution, efficacy, cytotoxicity, and immunogenicity *in vivo*. Another challenging aspect remains the poor correlation between *in vitro* and *in vivo* models.

These challenges should be addressed in future research. The need to apply delivery vectors, for example, can be determined by comparing the efficacy of siRNA with and without a delivery vector. Long-term effects should also be studied to determine if overcompensation of gene expression occurs. Likewise, comprehensive experiments (e.g., by using a DOE approach) are desired to elucidate the relationship between (un)favorable physicochemical properties of vectors and the efficacy, cytotoxicity, and immunogenicity *in vivo*. Furthermore, the lacking correlation between *in vitro* and *in vivo* models should be addressed by developing novel testing models (e.g., *ex vivo* models). *Ex vivo* models could have a

higher predictive value than *in vitro* models as they resemble target tissues more closely. Collectively, these future directions define the route to go: towards safer and more effective pulmonary administration of siRNA.

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